# Effect of water content and temperature on *Carica papaya* lipase catalyzed esterification and transesterification reactions

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*Reçu le 09/09/03*
*Accepté le 12/12/03*

## Introduction

The development of esterification and interesterification reactions catalyzed by microbial lipases in solvent free systems requires the study of the influence of the water activity ($a_w$) and reaction temperature. On one hand, $a_w$ is a major factor in a lipase-catalyzed esterification reactions in that enough water must be present to retain the enzymes active structure but an excess of water in the reaction medium results in the lipase catalyzing a competitive hydrolysis reaction. Accordingly, a study of $a_w$ and total water content must be done in defining the optimal reaction conditions that allow the enzyme to catalyze acyl transfer reactions while excluding hydrolysis. The best results for the majority of microbial lipase preparations are obtained over the $a_w$ range of 0.25 to 0.45, which corresponds to a water content of between 0.5% to 1% [1]. The optimal water content, however, can vary up to values as high as 11% [2, 3]. Lipases typically operate at ambient pressure and temperature but the optimal activity of a lipase is temperature dependent. With some exceptions, the optimum activity temperature for fungal and yeast lipases lies between 30°C and 50°C [4-9]. In contrast, the optimal activity temperature for bacterial lipases is between 37°C and 65°C with a majority being at 50-65°C [10, 11].
Over the past few years, a large body of work has been reported on reactions catalyzed by plant lipases [12-25]. From an industrial viewpoint, crude plant extracts are readily available and generally are low cost materials. Moreover, several plant lipases are obtained as naturally supported crude preparations [16]. Recent work in particular has pointed out that *Carica papaya* latex exhibits a powerful lipase activity for catalyzing hydrolysis [17, 18], esterification [19, 20] and interesterification type reactions [18, 21-24]. Optimal reaction conditions, however, regarding the affect of temperature and level of hydration of the crude enzyme preparation were not reported in detail.
To this end, the present paper reports the effect of water content and temperature on the acyl transfer activity of a crude *Carica papaya* preparation in a water and solvent free system. The purpose of the work was to demonstrate the affect of these factors on the nature of the reactions catalyzed by this plant lipase.

## Experimental procedures

### *Materials*

All chemicals used were reagent grade and purchased from Sigma (St Louis, MO, USA). The 1-butanol (Sigma), HPLC grade > 99.8% purity, initial water content < 0.03% was dried by using sodium sulfate before use. The initial water content of trilaurin (Sigma, grade > 99% purity)

was insignificant. A papaya latex, obtained by bleeding of unripe *C. papaya* fruits, was simply dried. This commercially-available preparation of "papain crude powder" (Ref P3375) was purchased from Sigma and served as the crude *C. papaya* preparation. Lipase hydrolysis activity was measured using a tributyrin emulsion at pH 8.0 at 50°C [24]. The initial water content of the biocatalyst preparation was determined as the weight lost after drying the crude latex at 103°C for 24 hours in an oven. The initial $a_w$ of the enzyme preparation was measured at 25°C with an $a_w$ meter (FA-ST/1 instrument, GBX Scientific Instrument, Romans, France).

## *Measurement of biocatalyst water sorption isotherm*

The moisture sorption of the enzyme preparation was determined at 25°C using a DVS-1 automated moisture sorption analyzer (Surface Measurement Systems Ltd., London, U.K.). A typical sample size of 20 mg was chosen as a suitable compromise between sensitivity, equilibration time and homogeneity of the latex preparation. Before sorption analysis the preparation was dried at $a_w$ near zero with a continuous flow of dry air of 0.1% relative humidity (RH). At 25°C the instrument has a working humidity range from 0% to 98% RH with a sensitivity of 0.1 microgram. The required humidities were generated by mixing dry and water saturated gases in the correct proportions using two mass flow controllers and one vapor humidifier (dry air, $N_2$; total flow rate of 200 mL/sec.). The instrument was run with a mass variation (dm/dt, m: mass, t: time) set at 0.002%/min to reach equilibrium. This means that the relative humidity remained constant until the mass variation of the latex preparation remained consistent at this threshold. Once this condition was reached the relative humidity of the system was raised to the next level.

## *Procedures for esterification reaction*

Lauric acid (1 mmol: 200 mg) and 1-butanol (20 mmol: 1480 mg) were mixed with a magnetic stirrer at 200 rpm in a closed vial (25 mL). The reaction was started by the addition of 170 mg crude *C. papaya* preparation (10% w/w of total substrates) and heated. To determine the optimal reaction temperature, the reaction was performed at several temperatures (22°C to 60°C) for 24 hours. To determine the thermal stability of the lipase, reactions were carried out at 55°C by using 170 mg crude *C. papaya* lipase that had been previously pre-heated at several temperatures (50°C to 100°C) for time periods varying from 15 min to 3 h. To study the effects of biocatalyst $a_w$ and water content on esterification yields, reactions were carried out at 55°C and started by adding 170 mg crude *C. papaya* lipase preparation that had been pre-equilibrated at several $a_w$ values. The crude latex was equilibrated in desiccators containing $P_2O_5$ ($a_w$ = 0.02) or different saturated salt solutions for at least two weeks at 25°C [26, 27]. The salts used ($a_w$ shown in parentheses) were LiCl ($a_w$ = 0.12), KAc ($a_w$ = 0.22), $MgCl_2$ ($a_w$ = 0.33), potassium carbonate ($a_w$ = 0.44), $Mg(NO_3)_2$ ($a_w$ = 0.55), LiAc ($a_w$ = 0.67) and KCl ($a_w$ = 0.86). Over the time course of the reactions, samples (25 µL) were removed from the reaction mixture. After dilution with 1 mL *n*-hexane, the reaction was stopped by filtering off the catalyst using a syringe filter (Millex 0.5 µm, Millipore, Guyancourt, France). An aliquot of the filtrate (100 µL) was added to a tube containing 1 mL *n*-hexane, and 0.5 µL of this mixture was analyzed by gas chromatography (GC). The non-esterified compounds and the synthesized butyl laurate were analyzed on a nonpolar capillary column (Rtx-1, 3 m 0.32 mm i.d.; Restek, Bellefonte, PA, USA). The GC was equipped with an on-column capillary injector and a flame-ionization detector (370°C). The carrier gas was helium at a flow rate of 5.5 mL/min. Analysis was carried out using the following oven temperature program: 90°C for 1 min, then heating at 10°C/min to 200°C and holding for 1 min.

## *Procedures for transesterification (alcoholysis) reaction*

Trilaurin (0.33 mmol; 214 mg) and 1-butanol (20 mmol; 1480 mg) were mixed with a magnetic stirrer at 200 rpm in a closed vial (25 mL). The reaction was started by adding 170 mg crude *C. papaya* lipase preparation and conducted at several reaction temperatures (22°C to 60°C) for 24 hours. To study the effects of biocatalyst $a_w$ and water content on transesterification yields, reactions were carried out at 55°C and started by adding 170 mg crude *C. papaya* lipase preparation that had been pre-equilibrated at several $a_w$ values as described above. Over the time course of the reaction, samples (25 µL) were removed from the reaction mixture. After dilution with 1 mL *n*-hexane, the reaction was stopped by filtering off the catalyst. An aliquot (100 µL) of the filtrate was added to a tube containing 2 mL *n*-hexane, and 0.5 µL of this mixture was analyzed by GC as described above. The oven conditions were: 90°C for 1 min, then heating at 20°C/min to 280°C, and holding for 6 min.

## Results and discussion

### *Intrinsic synthetic activity of* C. papaya *lipase*

First, an esterification reaction was carried out between pure lauric acid and 1-butanol in a solvent-free system at 55°C using a *C. papaya* lipase preparation (900 IU per g preparation) equilibrated at $a_w = 0.46$, which resulted in a water content of 3.9%. A similar reaction was carried out without the latex preparation (blank trial). Results of these experiments are shown in *figure 1*. Butyl laurate formation was observed in the blank trial but the reaction rate (8% conversion of lauric acid at 70 h) was slower than that observed in the presence of the latex (20% conversion). This could be explained by a thermal esterification of lauric acid to butyl laurate. The presence of a thermal autoreaction also was observed when the plant extract bromelain was used to catalyze esterification reactions [28]: in contrast to *C. papaya* latex, however the intrinsic lipase activity of bromelain is nil.

### *Effect of the temperature on esterification and transesterificat*ion

Esterification and transesterification reactions were carried out at temperatures from 30°C to 60°C. The effect of temperature was studied by determining the amount of ester formed after 24 hours of reaction (% conversion to butyl laurate). To discriminate between the synthesis activity of *C. papaya* lipase and thermal synthesis, at every temperature studied the rate of autocatalyzed ester synthesis was determined *(figure 2)*, and this rate corrected the enzyme catalyzed rate observed. The results of enzyme catalysis are then expressed as normalized synthesis rates as shown in *figure 3*, with 100% representing the maximum enzyme-catalyzed reaction yield at 24 h and 0% the corresponding yield of the thermal reaction. No thermal "autocatalysis" was observed in the transesterification between trilaurin and 1-butanol. Increasing the temperature from 30°C to 60°C resulted in a linear increase in the enzymatic synthesis of butyl laurate: normalized synthesis rates were 35%, 63% and 84% at 30°C, 40°C and 50°C, respectively. Maximum enzyme-catalyzed yields, which correspond to 9.8% and 14.6% conversion to butyl laurate at 24h in esterification and transesterification reactions respectively, were obtained at 55°C. It is interesting to note that the catalytic activity of the enzyme was still maximal at 60°C, which indicates the relative thermal stability of the *C. papaya* latex lipase [16]. Nevertheless, it is recommended to work at 55°C for industrial consideration (lower energy cost). In aqueous medium, the optimal temperature for the *C. papaya* lipase catalyzed hydrolysis of tributyrin was about 50°C but its hydrolytic activity decreased after 10 min reaction at 55°C [18]. Furthermore, the yields of the esterification and transesterification reactions as a function of the temperature were comparable despite water being produced in the former reaction but not the latter *(figure 3)*.

## *Thermal stability of* C. papaya *lipase*

An esterification reaction between lauric acid and 1-butanol was performed at 55°C in a micro-aqueous medium using the *C. papaya* lipase preparation after pre-incubation of the lipase at temperatures between 50°C to 100°C for time periods varying from 0 to 3 h. The effect of lipase pre-incubation was studied by determining the amount of butyl ester formed after 24 hours of reaction. The results were compared to butyl laurate synthesis obtained after 24 h using the non-incubated *C. papaya* lipase as the biocatalyst. Plotting the normalized enzyme synthesis rates against the pre-incubation condition showed that butyl ester yields are a function of pre-incubation conditions. This is illustrated in *figure 4*. Increasing the lipase pre-incubation temperature decreased the product yields. The decrease was most pronounced for pre-incubation temperatures above 80°C with the drop in catalytic activity being ≤ 10% for temperatures below 70°C. No thermal inactivation of the lipase was observed after 3 hours of pre-incubation at 50°C, as the product yield was still optimal. These results indicate a temperature stability of *C. papaya* lipase over the range of 50°C to 70°C. An important loss of lipase activity, however, occurred after 1 hour pre-incubation at temperatures ≥ 80°C (50% normalized rate corresponding to 7.5% conversion to butyl laurate). After incubation for one hour at 100°C the biocatalyst lost most of its activity as the yield of butyl laurate corresponded to that of the thermal reaction without enzyme (4.9% conversion to butyl laurate at 24 h).

## *Optimal hydration state for* C. papaya *lipase-catalyzed synthesis reactions*

The aqueous environment of a system strongly influences the biocatalytic activity of an enzyme preparation. For lipid synthesis, reactions performed with water-free substrates and in solvent-free systems, a minimal hydration of the lipase is needed to maintain its activity toward ester synthesis [29-31]. Because of this, in the present study the water was limited to that present in the *C. papaya* latex preparation. An optimal hydration level governed by the $a_w$ and water content of the latex, for which the best ester yields are obtained, does exist. In this regard, esterification and transesterification reactions were carried out at 55°C for 24 h by adding *C. papaya* lipase preparations pre-equilibrated at various $a_w$ values between 0 and 1. It is not required that substrates be equilibrated to desired water activities prior to reaction because their water content is insignificant as compared to the contribution of the lipase preparation [31, 32]. The yields of newly-formed ester at 24 h were calculated after subtracting the ester yields in a blank trial and expressed as normalized rates *(figure 5)*. This bell-shaped curve clearly shows the effect of $a_w$ on the *C. papaya* lipase activity in ester synthesis. The maximum product yield obtained with this biocatalyst was at an $a_w$ value of 0.22, both for esterification (19.7% conversion of lauric acid) and transesterification (29.3% conversion of trilaurin). $A_w$ values > 0.22 for the *C. papaya* lipase preparation resulted in a gradual decrease in product yields for esterification and transesterification and approached zero near an $a_w \sim$ of 0.7. The effect of $a_w$ on water content of the crude preparation also was studied by tracing the water sorption curve of the biocatalyst. First of all, the dried biocatalyst is a very hydrophilic preparation absorbing almost 40 g of water per 100 g of dry latex at an $a_w \sim 0.9$. The optimum $a_w$ value of 0.22 corresponds to a hydration of the crude *C. papaya* lipase powder of 2% water (2 g of water per 100 g of dry material). It is noteworthy to point out that this *a*w is located on the linear section of the sorption curve ($0.05 < a\text{w} \leq 0.35$) *(figure 5)*. Accordingly, this hydration level retains the lipase maximum activity in catalyzing both esterification and acyl transfer reactions while excluding hydrolysis. For *a*w values > 0.22 the excess water shifts the reaction towards hydrolysis rather than ester synthesis [1]. It is especially interesting to note that an *a*w value > 0.35 corresponds to the limit between water “highly adsorbed” to the dry latex and the “solvating water” absorbed through capillary and osmotic forces [33]. Drying the crude latex at 40°C in an oven confirmed the results of the water sorption curve: the “solvating

water" ($a_w \geq 0.35$) was easily eliminated in 3 hours; in contrast, it was difficult to decrease the water content below $a_w$ of 0.35 *(figure 6)*. Conditioning the *C. papaya* lipase preparation to an $a_w$ near 0.22 (which correspond to 2 g of water per 100 g of dry biocatalyst material) and maintaining the temperature at 55°C in the reaction system is very important for maximal lipase activity in catalyzing both the esterification and transesterification reactions. It was demonstrated that over the course of the reactions the catalytic activity of the *C. papaya* lipase preparation was dependent on *(i)* temperature and *(ii)* water content, to obtain maximum ester yields. One must consider, however, whether the optimal conditions reported in this paper can be applied to all ester syntheses and acyl transfer reactions catalyzed by *C. papaya* lipase in micro-aqueous and solvent-free systems.

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Illustrations

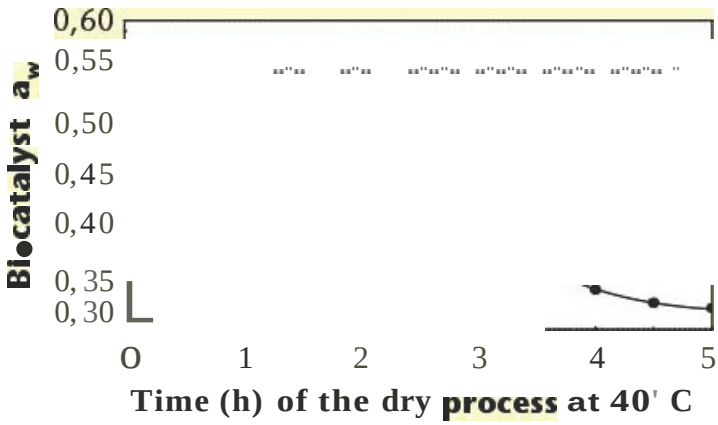


**Figure 6.** *Thermodynamic water activity ($a_{w)}$ of crude papain preparation during its dry process at 40°C in a closed oven.*